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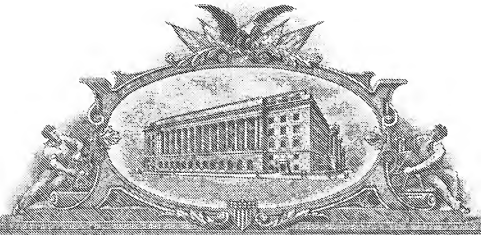

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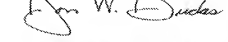

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Commissioner for Patents
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Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM

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Enclosed are:

(X) Specification in 21 pages.

(X) 8 sheets of drawings.

(X) The present application qualifies for small entity status under 37 CFR 1.27.

(X) A check in the amount of $80 to cover the filing fee is enclosed.

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CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM

Field of the Invention

[0001] The present invention relates to the fields of medicine and cell biology. In particular, the present invention relates to compositions comprising CXCR4 and SOX17 expressing definitive endoderm cells and methods of making, isolating and using such cells.

Background

[0002] Human embryonic stem (ES) cells or human embryonic germ (EG) cells were first isolated in culture without fibroblast feeders in 1994 (Bongso et al., 1994) and with fibroblast feeders (Hogan, 1997; Labosky et al., 1994a; Labosky et al., 1994b). Later, Thomson, Reubinoff and Shamblott established continuous cultures of human ES and EG cells using mitotically inactivated mouse feeder layers (Reubinoff et al., 2000; Shamblott et al., 1998; Thomson et al., 1998).

[0003] Human ES and EG cells (hESCs) offer unique opportunities for investigating early stages of human development as well as for therapeutic intervention in several disease states, such as diabetes mellitus and Parkinson's disease. For example, the use of insulin-producing β-cells derived from hESCs would offer a vast improvement over current cell therapy procedures which utilize cells from donor pancreases. Currently cell therapy treatments for diabetes mellitus, which utilize cells from donor pancreases, are limited by the scarcity of high quality islet cells needed for transplant. Cell therapy for a single Type I diabetic patient requires a transplant of approximately 8×10^8 pancreatic islet cells. (Shapiro et al., 2000; Shapiro et al., 2001a; Shapiro et al., 2001b). As such, at least two healthy donor organs are required for to obtain sufficient islet cells for a successful transplant. HESCs offer a source of starting material from which to develop substantial quantities of high quality differentiated cells for human cell therapies.

[0004] Two properties that make hESCs uniquely suited to cell therapy applications are pluripotence and the ability to culture for prolonged periods without

accumulation of genetic changes. Pluripotence is defined by the ability of hESCs to differentiate to derivatives of all 3 primary germ layers (endoderm, mesoderm, ectoderm) which, in turn, form all cell types of the mature organism. Although pluripotency imparts extraordinary utility upon hESCs, this property also poses unique challenges for the study and manipulation of these cells and their derivatives. Owing to the large variety of cell types that may arise in differentiating hESC cultures, the vast majority of cell types are produced at very low efficiencies. Additionally, success in evaluating production of any given cell type depends critically on defining appropriate markers. Achieving efficient, directed differentiation is of great importance for therapeutic application of hESCs.

[0005] In order to use hESCs in cell therapy, it would be useful to overcome the foregoing problems. For example, in order to achieve the level of cellular material required for islet cell transplant therapy, it would be useful to efficiently direct hESCs toward the pancreatic islet/ β-cell lineage at the very earliest stages of differentiation.

[0006] In addition to efficient direction of the differentiation process, it is also useful to achieve isolation of intermediate cell types along the differentiation path towards the pancreatic islet/ β-cell lineage. This allows a more definitive characterization of the cell type being isolated and permits further steps in the differentiation process to be derived entirely from the appropriate lineage precursor(s).

Summary of the Invention

[0007] One embodiment of the present invention is the discovery of a cell surface marker useful for the isolation and purification of definitive endoderm cells. A second embodiment is a method for using reagents which specifically bind to this receptor for the enrichment, isolation, separation or purification of definitive endoderm (DE).

[0008] Other embodiments of the present inventions are described with reference to the numbered paragraphs below:

[0009] 1. A mammalain cell composition comprising endodermal cells, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-

fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 10% of said endodermal cells.

[0010] 2. The cell composition of Paragraph 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 20% of said endodermal cells.

[0011] 3. The cell composition of Paragraph 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 40% of said endodermal cells.

[0012] 4. The cell composition of Paragraph 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 50% of said endodermal cells.

[0013] 5. The cell composition of Paragraph 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 70% of said endodermal cells.

[0014] 6. The cell composition of Paragraph 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 90% of said endodermal cells.

[0015] 7. A mammalain cell composition comprising endodermal cells, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 10% of said endodermal cells.

[0016] 8. The cell composition of Paragraph 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 20% of said endodermal cells.

[0017] 9. The cell composition of Paragraph 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 40% of said endodermal cells.

[0018] 10. The cell composition of Paragraph 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 50% of said endodermal cells.

[0019] 11. The cell composition of Paragraph 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 70% of said endodermal cells.

[0020] 12. The cell composition of Paragraph 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 90% of said endodermal cells.

Brief Description of the Drawings

[0021] Figure 1 is a bar chart showing the relative expression level of CXCR4 in differentiating hESCs under various media conditions.

[0022] Figure 2 A-D are bar charts that show how a panel of definitive endoderm markers share a very similar pattern of expression to CXCR4 across the same differentiation treatments displayed in Figure 1.

[0023] Figure 3 A-E are bar charts showing how markers for mesoderm (BRACHYURY, MOX1), ectoderm (SOX1, ZIC1) and visceral endoderm (SOX7) exhibit an inverse relationship to CXCR4 expression across the same treatments displayed in Figure 1.

[0024] Figure 4 A-C are micrographs that show the relative difference in SOX17 immunoreactive cells across three of the media conditions displayed in Figures 1-3.

[0025] Figure 5 A-C are flow cytometry dot plots that demonstrate the increase in $CXCR4^+$ cell number with increasing concentration of activin A added to the differentiation media.

[0026] Figure 6 is a bar chart showing gene expression from $CXCR4^+$ and $CXCR4^-$ cells isolated using fluoresence-activated cell sorting (FACS) as well as gene expression in the parent populations. This demonstrates that the $CXCR4^+$ cells contain essentially all the CXCR4 gene expression present in each parent population and the $CXCR4^-$ populations contain very little or no CXCR4 gene expression.

[0027] Figure 7 A-D are bar charts that show the $CXCR4^+$ cells isolated from the high dose activin A treatment (A100-CX+) are even further enriched for definitive endoderm markers than the parent population (A100).

[0028] Figure 8 A-D are bar charts that demonstrate the depletion of mesoderm (BRACHYURY, MOX1), ectoderm (ZIC1) and visceral endoderm (SOX7) gene expression in the CXCR4+ cells isolated from the high dose activin A treatment which is already suppressed in expression of these non-definitive endoderm markers.

Detailed Description

[0029] In accordance with some embodiments of the present invention, methods of producing definitive endoderm from stem cells, such as pluripotent stem cells, are disclosed. Stem cells used in these methods can include, but are not limited to, embryonic stem cells. Embryonic stem cells can be derived from the embryonic inner cell mass or from the embryonic gonadal ridges. Embryonic stem cells can originate from a variety of animal species including, but not limited to, various mammalian species including humans.

[0030] In some embodiments of the present invention, one or more growth factors are used in the differentiation process from stem cell to definitive endoderm cell. Such factors can include growth factors from the BMP subgroup of the TGFβ superfamily. For example, such factors include, but are not limited to Nodal, Activin A, Activin B, BMP4 and combinations thereof. The use of the growth factor Wnt3a is also contemplated.

[0031] Other aspects of the invention disclosed herein relate to compositions which comprise both stem cells and definitive endoderm cells. In some embodiments, such

compositions also include one or more growth factors. In other embodiments, some compositions described herein are substantially purified definitive endoderm cells.

[0032] In some embodiments of the present invention SOX17 antibodies can be used to isolate definitive endoderm cells in an isolated or substantially purified form. Methods known in the art, such as affinity-based or magnetic-based separation, can be used to obtain isolated or substantially purified preparations of definitive endoderm cells bound to the SOX17 antibody.

[0033] The compositions and methods described herein have several useful features. For example, the compositions and methods described herein are useful for modeling the early stages of human development. Furthermore, the compositions and methods described herein can also serve for therapeutic intervention in disease states, such as diabetes mellitus. For example, since definitive endoderm serves as the source for only a limited number tissues, it can be used in the development of pure tissue or cell types.

[0034] A crucial stage in early human development termed gastrulation occurs 2-3 weeks after fertilization. Gastrulation is extremely significant because it is at this time that the three primary germ layers are first specified and organized (Lu et al., 2001; Schoenwolf and Smith, 2000). The ectoderm is responsible for the eventual formation of the outer coverings of the body and the entire nervous system whereas the heart, blood, bone, skeletal muscle and other connective tissues are derived from the mesoderm. Definitive endoderm is defined as the germ layer that is responsible for formation of the entire gut tube which includes the esophagus, stomach and small and large intestines, and the organs which derive from the gut tube such as the lungs, liver, thymus, parathyroid and thyroid glands, gall bladder and pancreas (Grapin-Botton and Melton, 2000; Kimelman and Griffin, 2000; Tremblay et al., 2000; Wells and Melton, 1999; Wells and Melton, 2000). A very important distinction should be made between the definitive endoderm and the completely separate lineage of cells termed primitive endoderm. The primitive endoderm is primarily responsible for formation of extra-embryonic tissues, mainly the parietal and visceral endoderm portions of the placental yolk sac and the extracellular matrix material of Reichert's membrane.

[0035] During gastrulation, the process of definitive endoderm formation begins with a cellular migration event in which mesendoderm cells (cells competent to form

mesoderm or endoderm) migrate through a structure called the primitive streak. Definitive endoderm is derived from cells, which migrate through the anterior portion of the streak and through the node (a specialized structure at the anterior-most region of the streak). As migration occurs, definitive endoderm populates first the most anterior gut tube and culminates with the formation of the posterior end of the gut tube.

[0036] *In vivo* analyses of the formation of definitive endoderm, such as the studies in Zebrafish and *Xenopus* by Conlon et al., 1994; Feldman et al., 1998; Zhou et al., 1993; Aoki et al., 2002; Dougan et al., 2003; Tremblay et al., 2000; Vincent et al., 2003; Alexander et al., 1999; Alexander and Stainier, 1999; Kikuchi et al., 2001; Hudson et al., 1997 and in mouse by Kanai-Azuma et al., 2002 lay a foundation for how one might attempt to approach the development of a specific germ layer cell type in the culture dish using human embryonic stem cells. There are two aspects associated with in vitro ESC culture that pose major obstacles in the attempt to recapitulate development in the culture dish. First, organized germ layer or organ structures are not produced. The majority of germ layer and organ specific genetic markers will be expressed in a heterogeneous fashion in the differentiating hESC culture system. Therefore it is difficult to evaluate formation of a specific tissue or cell type due to this lack of organ specific boundaries. Almost all genes expressed in one cell type within a particular germ layer or tissue type are expressed in other cells of different germ layer or tissue types as well. Without specific boundaries there is considerably less means to assign gene expression specificity with a small sample of 1-3 genes. Therefore one must examine considerably more genes, some of which should be present as well as some that should not be expressed in the particular cell type of the organ or tissue of interest. Second, the timing of gene expression patterns is crucial to movement down a specific developmental pathway.

[0037] To further complicate matters, it should be noted that stem cell differentiation *in vitro* is rather asynchronous, likely considerably more so than *in vivo*. As such, one group of cells may be expressing genes associated with gastrulation, while another group maybe starting final differentiation. Furthermore, manipulation of hESC monolayers or embryoid bodies (EBs) with or without exogenous factor application may result in profound differences with respect to overall gene expression pattern and state of

differentiation. For these reasons, the application of exogenous factors must be timed according to gene expression patterns within a heterogeneous cell mixture in order to efficiently move the culture down a specific differentiation pathway.

[0038] Combining a method for isolation and purification of ontermediate cells types in the differentiation path is an effective way to deal with the above mentioned problems of heterogeneity and asynchrony.

PRODUCTION OF DEFINITIVE ENDODERM FROM STEM CELLS

[0039] The definitive endoderm cell cultures and compositions comprising definitive endoderm cells that are described herein can be produced from embryonic stem cells. A preferred method utilizes human embryonic stem cells (hESC) as the starting material for definitive endoderm production. The embryonic stem cells used in this method can be cells that originate from the embryonic inner cell mass or those obtained from embryonic gonadal ridges. Human stem cells can be maintained in culture in a pluripotent state without substantial differentiation using methods that are known in the art. Such methods are described, for example, in US Patent Nos. 5,453,357, 5,843,780 and 6,200,806, the disclosures of which are incorporated herein by reference in their entireties.

[0040] In some embodiments of the methods described herein, hESCs are maintained on a feeder layer. Any feeder layer which allows hESCs to be maintained in a pluripotent state can be used in the methods described herein. One commonly used feeder layer for the cultivation of human embryonic stem cells is a layer of mouse fibroblasts. More recently, human fibroblast feeder layers have been developed for use in the cultivation of hESCs (see US Patent Application No. 2002/0072117, the disclosure of which is incorporated herein by reference in its entirety). Alternative embodiments of the methods described herein permit the maintenance of pluripotent hESC without the use of a feeder layer. Such methods have been described in US Patent Application No. 2003/0175956, the disclosure of which is incorporated herein by reference in its entirety.

[0041] The human embryonic stem cells used herein are can be maintained in culture either with or without serum. In some embodiments, serum replacement is used. In other embodiments, serum free culture techniques, such as those described in US Patent

Application No. 2003/0190748, the disclosure of which is incorporated herein by reference in its entirety, are used.

[0042] Stem cells are maintained in culture in a pluripotent state by routine passage until it is desired that they be differentiated into definitive endoderm. In some embodiments, differentiation to definitive endoderm is achieved by providing to the stem cell culture a growth factor of the TGFβ superfamily in an amount sufficient to promote differentiation to definitive endoderm. Growth factors of the TGFβ superfamily which are useful for the production of definitive endoderm are selected from the Nodal/Activin or BMP subgroups. In some embodiments of the differentiation methods described herein, the growth factor is selected from the group consisting of Nodal, Activin A, Activin B and BMP4. Additionally, the growth factor Wnt3a is useful for the production of definitive endoderm cells. In certain embodiments of the present invention, combinations of any of the above-mentioned growth factors can be used.

[0043] With respect to some of the embodiments of differentiation methods described herein, the above-mentioned growth factors are provided to the cells so that the growth factors are present in the cultures at concentrations sufficient to promote differentiation of at least a portion of the stem cells to definitive endoderm. In some embodiments of the present invention, the above-mentioned growth factors are present in the cell culture at a concentration of at least about 10 ng/ml, at least about 25 ng/ml, at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, or at least about 1000 ng/ml.

[0044] In certain embodiments of the present invention, the above-mentioned growth factors are removed from the cell culture subsequent to their addition. For example, the growth factors can be removed within about one day, about two days, about three days, about four days, about five days, about six days, about seven days, about eight days, about nine days or about ten days after their addition. In a preferred embodiment, the growth factors are removed about four days after their addition.

[0045] Cultures of definitive endoderm cells can be grown in medium containing reduced serum or no serum. In certain embodiments of the present invention, serum

concentrations can range from about 0.1% v/v to about 20% v/v. In some embodiments, definitive endoderm cells are grown with serum replacement. In other embodiments, definitive endoderm cells are grown in the presence of B27. In such embodiments, the concentration of B27 supplement can range from about 0.2% v/v to about 20% v/v.

[0046] The progression of the hESC culture to definitive endoderm can be monitored by quantitating expression of marker genes characteristic of definitive endoderm as well as the lack of expression of marker genes characteristic of hESCs and other cell types. One method of quantitating gene expression of such marker genes is through the use of quantitative PCR (Q-PCR). Methods of performing Q-PCR are well known in the art. Other methods which are known in the art can also be used to quantitate marker gene expression. Marker gene expression can be detected by using antibodies specific for the marker gene of interest.

[0047] As described further in the Examples below, a reliable marker of definitive endoderm is the SOX17 gene. As such, the definitive endoderm cells produced by the methods described herein express the SOX17 marker gene. Other markers of definitive endoderm are MIXL1, GSC and HNF3b. In some embodiments of the present invention, definitive endoderm cells express the SOX17 marker gene at a level higher than that of the SOX7 marker gene, which is characteristic of visceral endoderm (see Table 1). Additionally, in some embodiments, expression of the SOX17 marker gene is higher than the expression of the OCT4 marker gene, which is characteristic of hESCs. In other embodiments of the present invention, definitive endoderm cells express the SOX17 marker gene at a level higher than the that of the AFP, SPARC or Thrombomodulin (TM) marker genes. In certain embodiments of the present invention, the definitive endoderm cells produced by the methods described herein do not express PDX1 (PDX1-negative).

[0048] In some embodiments of the present invention, the definitive endoderm cell cultures produced by the methods described herein are substantially free of cells expressing the OCT4, SOX7, AFP, SPARC, TM, ZIC1 or BRACH marker genes. With respect to cells in cell cultures, the term "substantially free of" means that the specified cell type is present in an amount of less than about 5% of the total number of cells present in the cell culture.

[0049] Using the methods described herein, compositions comprising definitive endoderm cells substantially free of other cell types can be produced. Alternatively, compositions comprising mixtures of hESCs and definitive endoderm cells can be produced. For example, compositions comprising at least 5 definitive endoderm cells for every 95 hESCs can be produced. In other embodiments, compositions comprising at least 95 definitive endoderm cells for every 5 hESCs can be produced. Additionally, compositions comprising other ratios of definitive endoderm cells to hESCs are contemplated.

[0050] In some embodiments of the present invention, definitive endoderm cells can be isolated by using an affinity tag that is specific for such cells. One example of an affinity tag specific for definitive endoderm cells is an antibody that is specific to a marker polypeptide that is present on the cell surface of definitive endoderm cells but which is not substantially present on other cell types that would be found in a cell culture produced by the methods described herein. In some embodiments, a SOX17 antibody is used as an affinity tag for the isolation of definitive endoderm cells. Methods for using antibodies for cell isolation are known in the art and such methods can be implemented for use with the antibodies and cells described herein. In one embodiment, the SOX17 antibody is attached to a magnetic bead then allowed to bind to definitive endoderm cells which have been enzymatically treated to reduce intercellular and substrate adhesion. The cell/antibody/bead complexes are then exposed to a movable magnetic field which is used to separate bead-bound definitive endoderm cells from unbound cells. Once the definitive endoderm cells are physically separated from other cells in culture, the antibody binding is disrupted and the cells are replated in appropriate tissue culture medium. In addition to the procedure just described, definitive endoderm cells can also be isolated by other means for cell isolation.

[0051] A preferred embodiment for purification of definitive endoderm cells would be use of a marker expressed on the cell surface of the cells specifically when they are produced as described herein. One such marker which is the subject of this invention is the chemokine receptor CXCR4. The principal role of the CXCR4 receptor bearing cells and its chemoattractant ligand SDF-1 in the adult are believed to be the migration of hematopoetic cells to the bone marrow, lymphocyte trafficking and the differentiation of various B cell and macrophage blood cell lineages [Kim, C., and Broxmeyer, H. J. Leukocyte Biol. 65, 6-15

(1999)]. The CXCR4 receptor also functions as a coreceptor for the entry of HIV-1 into T-cells [Feng, Y., et al. Science, 272, 872-877 (1996)]. In an extensive series of studies carried out by [McGrath, K.E. et al. Dev. Biology 213, 442-456 (1999)], the expression of the chemokine receptor CXCR4 and its unique ligand, SDF-1 [Kim, C., and Broxmyer, H., J. Leukocyte Biol. 65, 6-15 (1999)], were delineated during early development and adult life in the mouse. The CXCR4/SDF1 interaction in development became apparent when it was demonstrated that if either gene was disrupted in transgenic mice [Nagasawa et al. Nature, 382, 635-638 (1996)], Ma, Q., et al Immunity, 10, 463-471 (1999)] it resulted in late embryonic lethality. McGrath et al. demonstrated that CXCR4 is the most abundant chemokine receptor messenger RNA detected during early gastrulating embryos (E7.5) using a combination of RNase protection and in situ hybridization methodologies. In the gastrulating embryo, CXCR4/SDF-1 signaling appears to be mainly involved in inducing migration of primative-streak germlayer cells and is expressed on definitive endoderm, mesoderm and extraembryonic mesoderm present at this time. In E7.2-7.8 mouse embryos, CXCR4 and alpha-fetoprotein are mutually exclusive indicating a lack of expression in visceral endoderm [McGrath, K.E. et al. Dev. Biology 213, 442-456 (1999)].

[0052] In one embodiment of the present invention, definitive endoderm is isolated using an antibody based method that allows the definitive endoderm cells to be separated (FAC-sorting; magnetic bead separation, using an AB to CXCR4, or SDF-1 ligand coupled methods), from other non-definitive endoderm cells preferably after the hESC cultures are induced to differentiate towards the definitive endoderm lineage.

[0053] One description of a definitive endoderm cell based on the expression of marker genes would be, SOX17 high, MIXL1 high, AFP low, SPARC low, Thrombomodulin low, SOX7 low, CXCR4 high.

ISOLATION OF DEFINITIVE ENDODERM DERIVED FROM STEM CELLS

EXAMPLE 1

Chemokine receptor 4 (CXCR4) expression correlates with markers for definitive endoderm and not markers for mesoderm, ectoderm or visceral endoderm

[0054] ESCs can be induced to differentiate to the definitive endoderm germ layer by the application of cytokines of the TGFβ family and more specifically of the activin/nodal subfamily (United States Provisional Patent Application No. 60/502,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003, the disclosure of which is incorporated herein by reference in its entirety). We have previously shown that the proportion of fetal bovine serum (FBS) in the differentiation culture media effects the efficiency of DE differentiation from ESCs (see United States Provisional Patent Application No. 60/502,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003; see also United States Provisional Patent Application No. 60/566,293, entitled PDX1 EXPRESSING ENDODERM, filed April 27, 2004, the disclosures of which are incorporated herein by reference in their entireties). This effect is such that at a given concentration of activin A in the media, higher levels of FBS will inhibit maximal differentiation to DE. In the absence of exogenous activin A, differentiation of ESCs to the DE lineage is very inefficient and the FBS concentration has much milder effects on the differentiation process of ESCs.

[0055] hESCs were differentiated by growing in RPMI media (Invitrogen, Carlsbad, CA; cat# 61870-036) supplemented with 0.5%, 2.0% or 10% FBS and either with or without 100 ng/mL activin A for 6 days. In addition, a gradient ranging from 0.5% to 2.0% over the first three days of differentiation was also used in conjunction with 100 ng/mL of activin A. After the 6 days, replicate samples were collected from each culture condition and analyzed for relative gene expression by real-time quantitative PCR. The remaining cells were fixed for immunofluorescent detection of SOX17 protein.

[0056] The expression levels of CXCR4 varied dramatically across the 7 culture conditions used (Figure 1). In general, CXCR4 expression was high in activin A treated cultures (A100) and low in those which did not receive exogenous activin A (NF). In addition, among the A100 treated cultures, CXCR4 expression was highest when FBS concentration was lowest. There was a remarkable decrease in CXCR4 level in the 10% FBS

condition such that the relative expression was more in line with the conditions that did not receive activin A (NF).

[0057] Definitive endoderm is characterized by the expression of SOX17, GSC, MIXL1, and HNF3β. The relative expression of these four genes across the 7 differentiation conditions very closely mirrors that of CXCR4 (Figure 2A-D). This suggests the fact that CXCR4 is also a marker of DE.

[0058] The other two germ layer lineages can be distinguished from definitive endoderm by their expression of various markers. Early mesoderm expresses the genes BRACYURY and MOX1 while nascent neuro-ectoderm expresses SOX1 and ZIC1. Figures 3A-D demonstrate that the cultures which did not receive exogenous activin A are preferentially enriched for mesoderm and ectoderm gene expression and that among the activin A treated cultures, the 10% FBS condition also has increased levels of mesoderm and ectoderm marker expression. These patterns of expression are the inverse to that of CXCR4 and indicate that CXCR4 is not highly expressed in mesoderm or ectoderm derived from ESCs at this developmental time period.

[0059] Early during mammalian development differentiation to extra-embryonic lineages also occurs. Of particular relevance here is the differentiation of visceral endoderm that shares the expression of many genes in common with definitive endoderm, including SOX17 (Kanai-Azuma et al., 2002). To distinguish definitive endoderm from extra-embryonic visceral endoderm one must examine a marker that is distinct between these two. SOX7 represents a marker that is expressed in the visceral endoderm but not in the definitive endoderm lineage (Kanai-Azuma et al., 2002). Thus, culture conditions that exhibit robust SOX17 gene expression in the absence of SOX7 expression are likely to contain definitive and not visceral endoderm. It is shown in Figure 3E that SOX7 is highly expressed in cultures that did not receive activin A and also exhibits increased expression even in the presence of activin A when FBS is included at 10%. This pattern is the inverse of the CXCR4 expression pattern and suggests that CXCR4 is not highly expressed in visceral endoderm.

[0060] The relative number of SOX17 immunoreactive ($SOX17^+$) cells present in each of the differentiation conditions mentioned above was also determined. In

general, when hESCs are differentiated in the presence of high dose activin A and low FBS concentration (0.5% - 2.0%) $SOX17^+$ cells are ubiquitously distributed throughout the culture. When high dose activin A is used but FBS is included at 10% (v/v) the $SOX17^+$ cells appear at much lower frequency and always appear in rather isolated clusters rather than evenly distributed throughout the culture (Figure 4A&B). A further decrease in $SOX17^+$ cells is seen when no exogenous activin A is used and in these conditions the $SOX17^+$ cells also appear in clusters and these clusters are smaller and much more rare than found in the high activin A, low FBS treatment (Figure 4C). This demonstrates that the CXCR4 expression patterns not only correspond to DE gene expression but also to the number of committed DE cells in each condition.

EXAMPLE 2

Differentiation conditions that enrich for definitive endoderm increase the proportion of CXCR4 positive cells

[0061] The dose of activin A also effects the efficiency at which definitive endoderm can be derived from ESCs (United States Provisional Patent Application No. 60/502,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003, the disclosure of which is incorporated herein by reference in its entirety). This example demonstrates that increasing the dose of activin A increases the proportion of $CXCR4^+$ cells in the culture.

[0062] hESCs were differentiated in RPMI media supplemented with 0.5%-2% FBS (increased from 0.5% to 1.0% to 2.0% over the first 3 days of differentiation) and either 0, 10, or 100 ng/mL of activin A. After 7 days of differentiation the cells were dissociated in PBS without Ca^{2+}/Mg^{2+} containing 2% FBS and 2 mM (EDTA) for 5 minutes at room temperature. The cells were filtered through 35 um nylon filters, counted and pelleted. Pellets were resuspended in a small volume of 50% human serum/50% normal donkey serum and incubated for 2 minutes on ice to block non-specific antibody binding sites. To this, 1 uL of mouse anti-CXCR4 antibody (Abcam, cat# ab10403-100) was added per 50 uL (containing approximately 10^5 cells) and labeling proceeded for 45 minutes on ice. Cells were washed by adding 5 mL of PBS containing 2% human serum (buffer) and pelleted. A second wash with 5 mL of buffer was completed then cells were resuspended in 50 uL buffer

per 10^5 cells. Secondary antibody (FITC conjugated donkey anti-mouse; Jackson ImmunoResearch, cat# 715-096-151) was added at 5 ug/mL final concentration and allowed to label for 30 minutes followed by two washes in buffer as above. Cells were resuspended at $5x10^6$ cells/mL in buffer and analyzed and sorted using a FACS Vantage (Beckton Dickenson) by the staff at the flow cytometry core facility (The Scripps Research Institute). Cells were collected directly into RLT lysis buffer (Qiagen) for subsequent isolation of total RNA for gene expression analysis by real-time quantitative PCR.

[0063] The number of $CXCR4^+$ cells as determined by flow cytometry is observed to increase dramatically as the dose of activin A is increased in the differentiation culture media (Figure 5A-C). The $CXCR4^+$ cells are those falling within the R4 gate and this gate was set using a secondary antibody-only control for which 0.2% of events were located in the R4 gate. The dramatically increased numbers of $CXCR4^+$ cells correlates with a robust increase in DE gene expression as activin A dose is increased (Figure 7A-D).

EXAMPLE 3

Isolation of CXCR4 positive cells enriches for definitive endoderm gene expression and depletes cells expressing markers of mesoderm, ectoderm and visceral endoderm.

[0064] The $CXCR4^+$ and $CXCR4^-$ cells identified in Example 2 above were collected and analyzed for relative gene expression and the gene expression of the parent populations was determined simultaneously.

[0065] The relative levels of CXCR4 gene expression are dramatically increased with increasing dose of activin A (Figure 6). This correlates very well with the activn A dose-dependent increase of $CXCR4^+$ cells (Figure 5A-D). It is also clear that isolation of the $CXCR4^+$ cells from each population accounts for nearly all of the CXCR4 gene expression in that population. This demonstrates the efficiency of the FACS method for collecting these cells.

[0066] Gene expression analysis reveals that the $CXCR4^+$ cells contain not only the majority of the CXCR4 gene expression, but they also contain almost all of the gene expression for markers of definitive endoderm. As shown in Figure 7A-D, the $CXCR4^+$ cells

are further enriched over the parent A100 population for SOX17, GSC, HNF3B, and MIXL1. In addition, the $CXCR4^-$ fraction contains very little gene expression for these DE markers. Moreover, the $CXCR4^+$ and $CXCR4^-$ populations display the inverse pattern of gene expression for markers of mesoderm, ectoderm and extra-embryonic endoderm. Figure 8A-D shows that the $CXCR4^+$ cells are depleted for gene expression of BRACHYURY, MOX1, ZIC1, and SOX7 relative to the A100 parent population. This A100 parent population is already low in expression of these markers relative to the low dose or no activin A conditions. These results suggests that the isolation of $CXCR4^+$ cells from hESCs differentiated in the presence of high activin A yields a population that is highly enriched for and substantially pure definitive endoderm.

References

[0067] Numerous literature and patent references have been cited in the present application. All references cited are incorporated by reference herein in their entireties.

[0068] For some references, the complete citation is in the body of the text. For other references the citation in the body of the text is by author and year, the complete citation being as follows:

[0069] D'Amour, et al. (2003) U.S. Provisional Patent Application No. 60/532,004

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[0071] Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. (2004) Development of definitive endoderm from embryonic stem cells in culture. Development. *131*,1651-62

[0072] McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. (1999) Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol. *213*, 442-56.

[0073] Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635-638.

[0074] Ma, Q., Jones, D., and Springer, T. A. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity *10*, 463-471.

[0075] Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science *272*, 872-877.

[0076] Kim, C. H., and Broxmeyer, H. E. (1999). Chemokines: signal lamps for trafficking of T and B cells for development and effector function. J Leukoc Biol *65*, 6-15.

WHAT IS CLAIMED IS:

1. A mammalain cell composition comprising endodermal cells, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 10% of said endodermal cells.

2. The cell composition of Claim 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 20% of said endodermal cells.

3. The cell composition of Claim 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 40% of said endodermal cells.

4. The cell composition of Claim 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 50% of said endodermal cells.

5. The cell composition of Claim 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 70% of said endodermal cells.

6. The cell composition of Claim 1, wherein the expression of a marker selected from the group consisting of CXCR4 and SOX17 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 90% of said endodermal cells.

7. A mammalain cell composition comprising endodermal cells, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 10% of said endodermal cells.

8. The cell composition of Claim 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 20% of said endodermal cells.

9. The cell composition of Claim 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 40% of said endodermal cells.

10. The cell composition of Claim 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 50% of said endodermal cells.

11. The cell composition of Claim 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 70% of said endodermal cells.

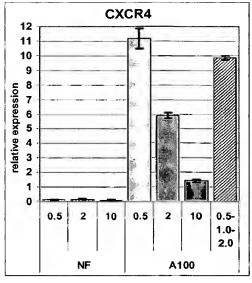
12. The cell composition of Claim 7, wherein the expression of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of alpha-fetoprotein (AFP), Thrombomodulin (TM) and SOX7 in at least about 90% of said endodermal cells.

CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM

Abstract of the Disclosure

Disclosed herein are compositions of definitive endoderm cells and substantially purified definitive endoderm cells. Also disclosed herein are methods for enriching and isolating definitive endoderm cells from other cell types.

Figure 1


CXCR4
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11
10
9
8
7
6
5
4
3
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2
10
0.5
2
10
0.5-
1.0-
2.0
NF
A100

Figure 2

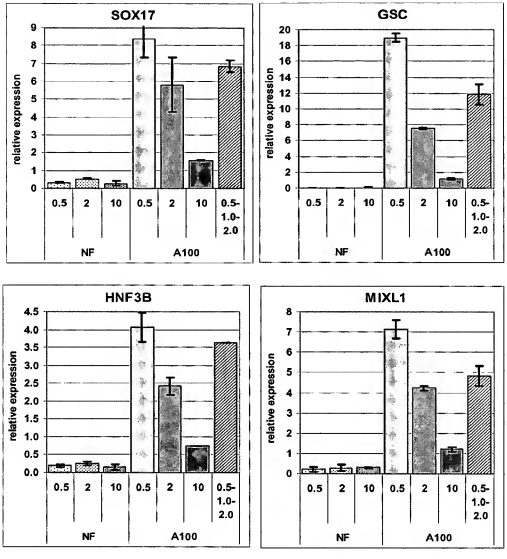
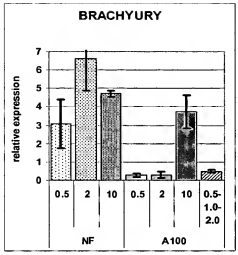

SOX17
relative expression
0.5
2
10
0.5
2
10
0.5-1.0-2.0
NF
A100
GSC
relative expression
0.5
2
10
0.5
2
10
0.5-1.0-2.0
NF
A100
HNF3B
relative expression
0.5
2
10
0.5
2
10
0.5-1.0-2.0
NF
A100
MIXL1
relative expression
0.5
2
10
0.5
2
10
0.5-1.0-2.0
NF
A100

Figure 3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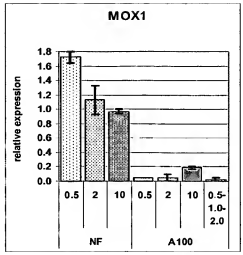
NF
A100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relative expression
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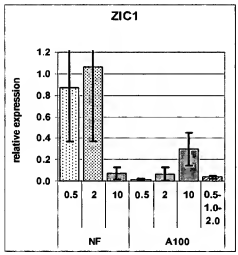
10
0.5
2
10
0.5-1.0-2.0
NF
A100


ZIC1
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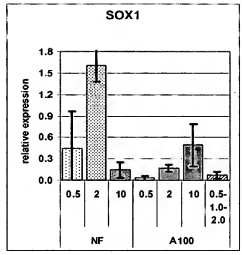
0.5
2
10
0.5-1.0-2.0
NF
A100


SOX1
relative expression
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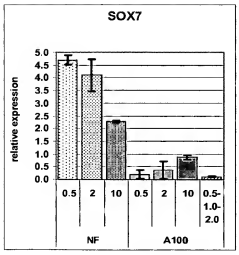
2
10
0.5
2
10
0.5-1.0-2.0
NF
A100


SOX7
relative expression
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2
10
0.5
2
10
0.5-1.0-2.0
NF
A100

Figure 4

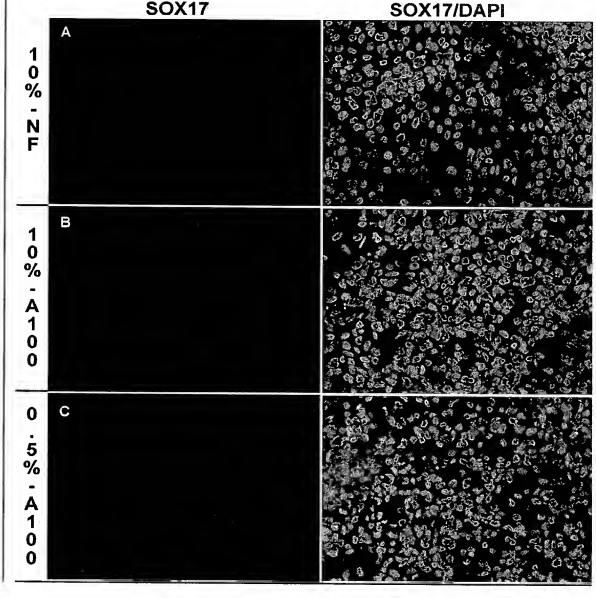


Figure 5

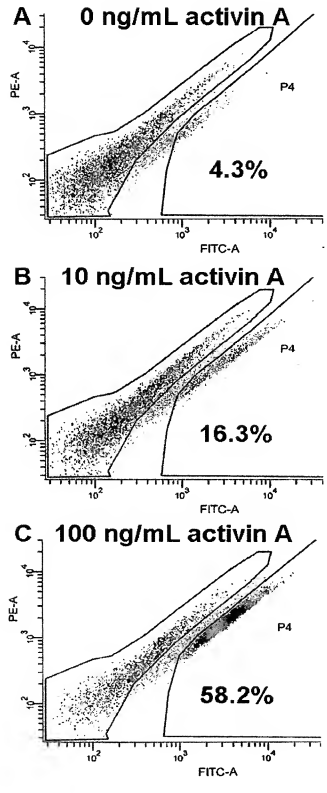
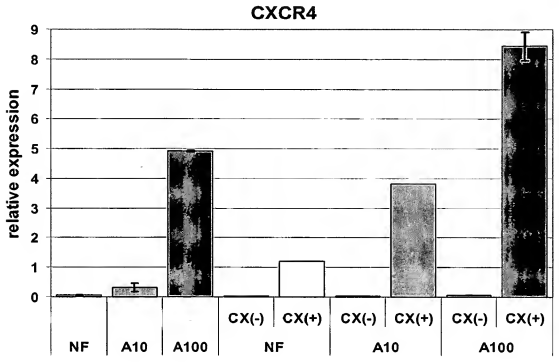

A
0 ng/mL activin A
P4
4.3%
PE-A
FITC-A
B
10 ng/mL activin A
P4
16.3%
PE-A
FITC-A
C
100 ng/mL activin A
P4
58.2%
PE-A
FITC-A

Figure 6


CXCR4
relative expression
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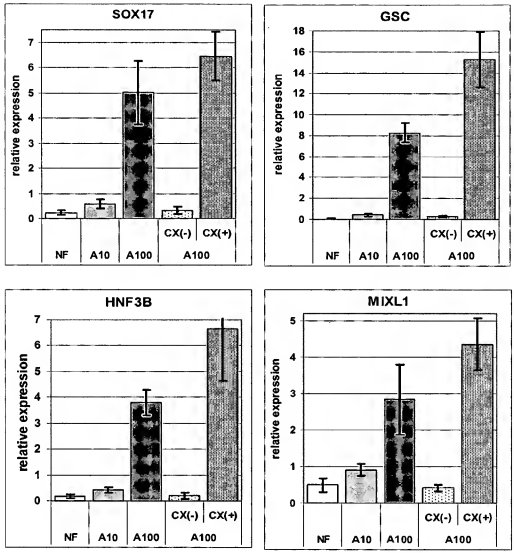
8
7
6
5
4
3
2
1
0
CX(-)
CX(+)
CX(-)
CX(+)
CX(-)
CX(+)
NF
A10
A100
NF
A10
A100

Figure 7


SOX17
relative expression
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1
2
3
4
5
6
7
NF
A10
A100
CX(-)
CX(+)
A100
GSC
relative expression
0
2
4
6
8
10
12
14
16
18
NF
A10
A100
CX(-)
CX(+)
A100
HNF3B
relative expression
0
1
2
3
4
5
6
7
NF
A10
A100
CX(-)
CX(+)
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MIXL1
relative expression
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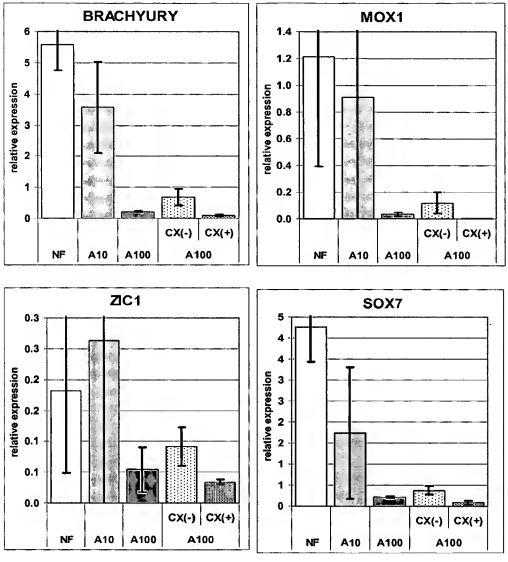
1
2
3
4
5
NF
A10
A100
CX(-)
CX(+)
A100

Figure 8


BRACHYURY
relative expression
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1
2
3
4
5
6
NF
A10
A100
CX(-)
CX(+)
A100
MOX1
relative expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
NF
A10
A100
CX(-)
CX(+)
A100
ZIC1
relative expression
0.0
0.1
0.1
0.2
0.2
0.3
0.3
NF
A10
A100
CX(-)
CX(+)
A100
SOX7
relative expression
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1
1
2
2
3
3
4
4
5
NF
A10
A100
CX(-)
CX(+)
A100